Biochemical analysis of the interaction between elongation factor 1α and α/β-tubulins from a ciliate, *Tetrahymena pyriformis*

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Abstract The interaction between elongation factor 1α (EF-1α) and α/β-tubulins has been analyzed in vivo and in vitro. An association of both α- and β-tubulins with EF-1α in the lysate of *Tetrahymena pyriformis* was detected by co-immunoprecipitation analysis. In contrast, in vitro biomolecular interaction analysis with glutathione *S*-transferase (GST) fusion proteins revealed that GST-β-tubulin, but not GST-α-tubulin, can bind to GST-EF-1α. Two β-tubulin binding sites have been identified to reside in the domains I and III of EF-1α. In addition, β-tubulin itself seems to have two distinct interaction sites for each of the domains. Since domain II of EF-1α did not interact with β-tubulin, we have re-evaluated the phylogenetic status of ciliates using EF-1α sequences devoid of domain II. The phylogenetic tree thus obtained was significantly different from that inferred from the whole sequence of EF-1α, suggesting the presence of functional constraints on the molecular evolution of EF-1α.

Key words: Elongation factor; Tubulin; Molecular evolution; Phylogeny; *Tetrahymena pyriformis*

1. Introduction

Elongation factor 1α (EF-1α) plays an essential role in translation of mRNA on ribosomes of eukaryotic cells [1]. EF-1α is functionally homologous to the prokaryotic factor EF-Tu and structurally conserved throughout evolution, and therefore it has been often used for phylogenetic studies [2]. Recently, an extensive phylogenetic study on EF-1α has been performed with EF-1α sequences from 15 ciliates [3]. Ciliophora, which consist of over 7000 species of ciliates, are represented as a monophyletic phylum on the phylogenetic tree of rRNA genes [4]. In the phylogenetic tree of EF-1α, however, Ciliophora do not show this monophyletic property; ciliates are divided into several branches in the tree [3].

On the other hand, EF-1α has been reported to interact with cytoskeletal proteins, such as tubulin and actin, in several organisms (for review, see [5]). For example: (1) a 51-kDa protein that is associated with the mitotic apparatus in sea urchin eggs has been identified as EF-1α [6]; (2) EF-1α has been shown to have microtubule-severing ability in *Xenopus* eggs [7]; (3) an actin binding protein of 50 kDa that bundles filamentous (F)-actin has been identified as EF-1α in *Dictyostelium discoideum* [8]; and (4) in a ciliate, *Tetrahymena*, EF-1α has been reported to bind to F-actin [9] and also to the 14-nm filament protein that has a dual function as citrate synthase and cytoskeletal protein [10]. However, the biochemical significance of the association between EF-1α and cytoskeletal proteins has not been elucidated.

Ciliates have a unique cytoskeletal organization that is mainly composed of microtubules. It has been shown that tubulin has a low evolutionary rate in ciliates [11]. This may be due to the functional constraint generated by the necessity of interacting with more than 200 proteins in the axoneme [11]. This strong constraint on tubulin may have influenced the evolution of the interacting partners as well. In this study, we have analyzed the association between EF-1α and α/β-tubulins from *Tetrahymena pyriformis* and found that EF-1α directly binds to β-tubulin but not to α-tubulin. We have also identified the domains in EF-1α responsible for β-tubulin binding, and re-evaluated the phylogenetic status of Ciliophora based on the sequences of the β-tubulin binding domains. The phylogenetic tree thus obtained was significantly different from that inferred from the whole sequence of EF-1α and seemed to resume the monophyletic property of Ciliophora in most of the ciliates.

2. Materials and methods

2.1. Chemicals and antibodies

Reagents of analytical grade were purchased from Sigma, Merck or Nacalai (Kyoto) unless otherwise noted. Anti-α-tubulin (DM1A) and anti-β-tubulin (DM1B) monoclonal antibodies against chicken tubulins [12] were purchased from Amersham. Anti-glutathione *S*-transferase (GST) antibody was kindly provided by Dr. Sato (Kobe University).

2.2. Cell culture and preparation of EF-1α

The GL strain of *T. pyriformis* was cultured in 1.5% proteose peptone (Difco) at room temperature until early stationary phase. Cultivated cells (2 liters) were harvested by centrifugation at 200×*g* for 1 min, washed once with Volvic water and resuspended in TME buffer (20 mM Tris-maleate, pH 7.5, 3 mM EDTA) supplemented with 10% glycerol and protease inhibitors (10 mM benzamidine-HCl and 1 mM phenylmethylsulfonyl fluoride). The cell suspension was transferred to a Potter homogenizer equipped with a Teflon pestle (0.15 mm clearance), and left on ice for 30–60 min until the cells were completely immobilized. Subsequent procedures were performed at 4°C. The cells were broken by 50–80 strokes of the pestle attached to a motor-driven shaft (500 rpm). The extent of cell breakage was monitored by phase-contrast microscopy. The homogenate was centrifuged at 12 000×*g* for 20 min, and the supernatant was recovered as the whole lysate. The protein concentration of this fraction was determined by the absorbance at 280 nm. The whole lysate was subjected to a phospho-

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Abbreviations: EF-1α, elongation factor 1α; GST, glutathione *S*-transferase

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cellulose (Sigma) column (1×5 cm) equilibrated with TME buffer. After washing the column with 15 ml of TME buffer, EF-1α was eluted with 10 ml of 0.6 M NaCl in TME buffer. The eluted fraction was determined for protein content by absorbance at 280 nm and then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE, see below) [13]. The gel was stained with Coomassie brilliant blue R-250 (Merck), and a major band migrating at 50 kDa was cut out from the gel. This band was identified to be EF-1α by amino acid sequence analysis (Ariad Protein Sequencing Service, USA).

2.3. Preparation of anti-EF-1α antibody

Anti-EF-1α polyclonal antibody was raised in a rabbit by injecting 100 μg of EF-1α in polyacrylamide gel that was emulsified with Freund's complete adjuvant. The second, third and fourth injections (100 μg each) were made at 2-week intervals. Fifty days after the first injection, the last boost was done, and the rabbit was bled 4 days after the last injection. The blood was kept overnight at 4°C and decanted after centrifugation. The obtained antiserum was stored at −20°C.

2.4. Immunoprecipitation of EF-1α

The whole lysate of *T. pyriformis* was dispensed into 500-μl aliquots and mixed with 15 μl of anti-EF-1α antiserum. A control sample was also prepared by mixing the whole lysate (500 μl) with 15 μl of pre-immune serum from the rabbit that raised the antibody. Mixtures were incubated at 4°C overnight with gentle shaking, and 75 μl (as a packed volume) of protein A-Sepharose beads (Sigma) in TME buffer was added to the mixture and incubated at 4°C for 2 h. Immunoprecipitates were washed four times with TME buffer and then suspended in 20 μl of the SDS sample buffer [13]. The SDS samples were denatured by boiling for 3 min.

2.5. SDS-PAGE and immunoblotting

SDS-PAGE was conducted according to the method of Laemmli [13], using 1.5 mm thick, 12 cm long, 10% polyacrylamide gels (Hoefer SE-250). Separated proteins were stained with Coomassie brilliant blue or transferred to a nitrocellulose membrane (BA85, Schleicher and Schuell). The membrane was blocked for 1 h in 0.3% (w/v) bovine serum albumin in PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4) and incubated with 0.3 μg/ml of monoclonal anti-tubulin antibody DM1A or DM1B which was diluted with PBS. After washing with PBS, membranes were incubated with the second antibody at room temperature for 1 h. The peroxidase-labeled secondary antibody (sheep anti-mouse IgG from Diagnostics Pasteur, France) was used at 0.6 μg/ml in PBS, and peroxidase was detected with the Enhanced Chemiluminescence Kit (ECL, Amersham).

2.6. GST fusion proteins

Escherichia coli (DH5α) cells were used for plasmid propagation and preparation of recombinant proteins. The vector pGEX-2T (Pharmacia) was used for the construction of GST fusion proteins, and insert DNAs were prepared by polymerase chain reaction (PCR). Genomic DNA of *T. pyriformis* was prepared as described [3], and plasmids, αTT and βTT1, containing the *T. pyriformis* α- and β-tubulin genes, respectively, were kindly provided by Dr. Soares (Instituto Gulbenkian de Ciencia, Portugal) [14]. PCR primers used were: forward primers for EF-1α and EF-1α domains II and III, 5′-CGGGATCCATGGCTAGAGGTGATAA-3′, 5′-AAAGGATCCGTTGACAAGCCCCTCAGA-3′ and 5′-TCCGGATCCAAGAACGACCCCGCCAAA-3′; reverse primers for EF-1α and EF-1α domains I and II, 5′-CGGGATCCTCATTTGTCCTTCTTTTC 3′, 5′-TCTGGATCCTCAGTCAACAGGTCTTTTGGG 3′ and 5′-GGGGGATCCTCAGGCATCGGAAGCGACATT-3′; forward primers for α- and β-tubulins, 5′-CGGGATCCATGAGAGAAGTTATTTC-3′ and 5′-CGGGATCCATGAGAGAAATCGTTCACATC**CAG**-3′; reverse primers for α- and β-tubulins, 5′-CGGGATCCGAATCAAGCAAATCAGTA-3′ and 5′-CGGGATCCTTTTATTCAGTTTCCTCAGTTTTC-3′, respectively. Underlined nucleotides indicate the *Bam*HI site at which PCR products were ligated to the vector. Nucleotides in bold represent a codon for glutamine. In *Tetrahymena* cells, TAA and TAG stop codons are suppressed and used as glutamine codons [15]. There were two TAG codons to be considered: one in EF-1α at glutamine-121 and the other in β-tubulin at glutamine-8. Although the DH5α strain of *E. coli* has *supE*, an amber (TAG) suppressor that inserts glutamic acid, we wanted to change these TAG codons to fit the normal codon usage of *E. coli*. Thus, the TAG codon in β-tubulin was replaced by a glutamine codon, **CAG**, in the sense primer listed above. In the case of EF-1α, we used a mutated antisense primer, 5′-TCGAATTCACC**CTG**GGGGGGAAGCAAT-3′, in which the original TAG codon is replaced by the glutamine codon, **CAG**. Since an *Eco*RI site (underlined) is near the mutation site, we used this site to ligate the mutant fragment to the rest of the wild-type EF-1α and reconstituted a full-length mutant. The DNA fragment corresponding to domain I of EF-1α was then produced by PCR using this mutant clone as a template. The boundaries between the domains were determined by computer modeling (QUANTA97) based on the published crystal structure of EF-Tu of *E. coli* [16] as well as the amino acid sequence alignment between EF-Tu and EF-1α. All the constructs were confirmed for their orientation and sequence by nucleotide sequencing.

E. coli cells harboring each of the constructs were grown in L-broth at 37°C, and the synthesis of fusion proteins was induced by adding 0.15 mM isopropyl-β-D-thiogalactoside for 2 h at 25°C. Cells were lysed by freezing-thawing in lysis buffer (20 mM Tris-HCl, pH 8.0, 10 mM EDTA, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin) supplemented with 1 mg/ml lysozyme (Seikagakukogyo, Japan). The crude lysate was recovered by centrifugation at 31 000×*g* for 20 min and mixed with 100 ml of 50% (w/v) glutathione-Sepharose (Pharmacia). After washing the Sepharose beads with lysis buffer, GST fusion proteins were eluted with 10 mM glutathione in 50 mM Tris-HCl (pH 9.5). The purified GST fusion proteins were analyzed by SDS-PAGE and immunoblotting with anti-GST antibody.

2.7. Real-time biomolecular interaction analysis

Biomolecular interaction analysis using the surface plasmon resonance technology was performed by BIACORE 1000 (Pharmacia). HBS buffer (10 mM HEPES-NaOH pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20) was used as a flow buffer, and the flow rate was kept at 5 μl/min. The temperature was maintained at 25°C. Streptavidin-linked dextran matrix (SA5) was used for immobilization of biotin-conjugated ligands. Biotinylation of the ligand proteins was carried out by incubating 10–60 μg of GST fusion proteins with 50 mg of sulfosuccinimidobiotin (Pierce) in 100 μl of 500 mM sodium phosphate buffer (pH 7.0) containing 1% dimethylsulfoxide at 4°C for 2 h. Then the mixture was applied to a PD-10 gel filtration column (Pharmacia) equilibrated with HBS buffer (pH 7.4), and fractions of 0.3 ml were collected. Proteins were monitored by the absorbance at 595 nm using Protein Assay Mixture (Bio-Rad), and peak fractions were pooled and stored at −20°C until use. GST-tubulin (40 μg/ml GST-α-tubulin or 67 μg/ml GST-β-tubulin) in HBS buffer was immobilized onto the surface of an SA5 chip for 7 min at 5 μl/min. Immobilized proteins gave resonance units (RU) of 808 and 1360 RU for GST-α-tubulin and GST-β-tubulin, respectively. Association and dissociation of GST fusion EF-1α proteins onto the α- or β-tubulin-immobilized surface were measured in continuous HBS buffer flow, and the surface was regenerated by washing with 50 mM HCl for 1 min followed by washing with HBS buffer after each binding experiment. Kinetic constants were determined using the BIA evaluation 2.1 (Pharmacia Biosensor AB).

2.8. Phylogenetic analysis

The tree was constructed with the neighbor-joining (NJ) distance program [17] with the NJ program of the MUST package, version 1.0 [18]. Eight phyla containing 44 species were used: Diplomonadida (*Hexamita inflata*, *Giardia lamblia*), Sporozoa (*Plasmodium falciparum*, *Cryptosporidium parvum*), Mycetozoa (*Dictyostelium discoideum*, *Physarum polycephalum*, two *Planoprotostelium aurantium*), Euglenozoa (*Euglena gracilis*, *Leishmania braziliensis*, *Trypanosoma brucei*, two *Trypanosoma cruzi*), Chlorobionta (*Daucus carota*, *Glycine max*, *Arabidopsis thaliana*, *Zea mays*, *Oryza sativa*), Metazoa (*Caenorhabditis elegans*, *Drosophila melanogaster*, two *Homo sapiens*), Fungi (*Neurospora crassa*, *Schizosaccharomyces pombe*, *Mucor racemosus*) and 19 species in Ciliophora used in [3]. All these sequences were manually aligned using the ED program of the MUST package [18] and an alignment of 393 positions was obtained. It contained the regions 22–157, 160–185, 186–214, 215–305, 306–336, 337–343, 344–379, 380–389 and 390–416 of the EF-1α molecules (following the numbering of the *T. pyriformis* EF-1α sequence). The phylogenetic tree was also inferred from the EF-1α sequence lacking domain II, 233–318 (*T. pyriformis* EF-1α sequence). The bootstrap proportions

were computed with the NJBOOT program of the MUST package [18].

3. Results

3.1. Co-immunoprecipitation of α/β-tubulins with EF-1α

We first examined whether EF-1α is associated with α/β-tubulins in *Tetrahymena* cells. In Fig. 1, the whole lysate of *Tetrahymena* cells was prepared and immunoprecipitated with anti-EF-1α antibody. The immunoprecipitates were then separated by SDS-PAGE and analyzed by immunoblotting with either anti-α-tubulin (Fig. 1A) or anti-β-tubulin antibody (Fig. 1B). In both cases, an immunoreactive protein that migrates at around 50 kDa was clearly detected (lane 1), while no significant band was found in the immunoprecipitates prepared with pre-immune serum (lane 2). The appearance of faint and cloudy bands in lanes 1 and 2 is probably due to cross-reactivity of the second antibody with a large amount of IgG heavy chain in the samples. Although the presence of IgG heavy chain diminished the sharpness of the immunoreactive bands in lane 1, each protein band showed a similar mobility to the original α- or β-tubulin in the lysate (lane 3). These results strongly suggest that EF-1α is in association with α- and β-tubulins in the lysate of *Tetrahymena* cells. It is not clear, however, whether EF-1α directly binds to both α- and β-tubulin monomers or to the oligomeric form of the tubulins, i.e. fragments of microtubules.

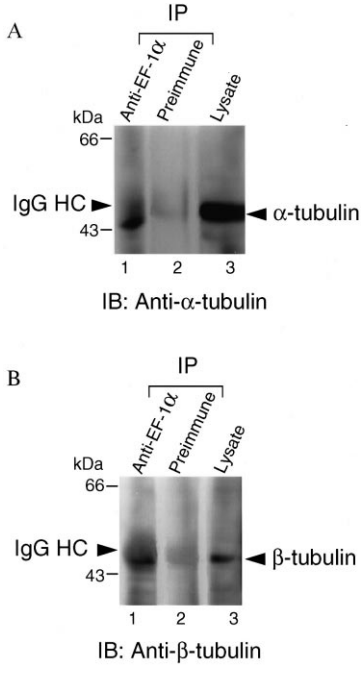

Fig. 1. Association of EF-1α with α/β-tubulin in *T. pyriformis*. Whole lysate (0.5 mg protein) prepared from *T. pyriformis* was immunoprecipitated (IP) with either anti-EF-1α antibody (lane 1) or pre-immune antibody (lane 2) as described in Section 2.4. The immunoprecipitates were separated by SDS-PAGE and analyzed by immunoblotting (IB) with either anti-α-tubulin (A) or anti-β-tubulin (B) antibody as described in Section 2.5. In lane 3 of each panel, the whole lysate (0.5 mg protein) was directly analyzed by immunoblotting with anti-tubulin antibodies. The positions of the IgG heavy chain (HC), α- and β-tubulins are indicated. Molecular size markers used are bovine serum albumin (66 kDa) and bovine muscle actin (43 kDa).

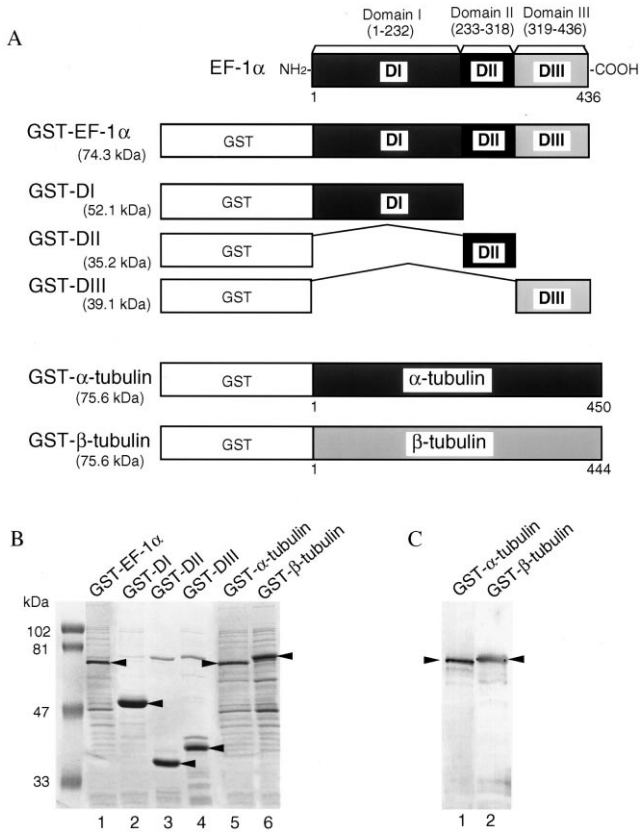

Fig. 2. Preparation of *T. pyriformis* EF-1α, α-tubulin, β-tubulin and three domains of EF-1α as GST fusion proteins. Schematic structures (A) and Coomassie brilliant blue (CBB)-stained SDS gel (B) of the preparations of GST fusion proteins used in this study are shown. Preparation of plasmid DNAs for these constructs and their bacterial expression and purification is described in Section 2.6. In panel A, the calculated molecular mass of each GST fusion protein is shown in parentheses. Three domains of EF-1α, domain I (DI), domain II (DII) and domain III (DIII), are shown with their amino acid residue numbers. In panel B, 5 μg of each preparation of GST fusion protein was analyzed by SDS-PAGE. The positions of GST fusion proteins are marked by arrowheads. Prestained molecular size markers (Bio-Rad) are phosphorylase B (102 kDa), bovine serum albumin (81 kDa), ovalbumin (47 kDa), and carbonic anhydrase (33 kDa). In panel C, purified GST-α-tubulin (lane 1) and GST-β-tubulin (lane 2) were analyzed by immunoblotting (IB) with anti-GST antibody.

3.2. EF-1α directly binds to β-tubulin but not to α-tubulin

To test the direct binding of EF-1α to α/β-tubulins in a purified system, we have expressed recombinant EF-1α and α/β-tubulins in *E. coli* as GST fusion proteins (Fig. 2). We also attempted to identify the tubulin binding domain(s), if any, in the EF-1α molecule. Thus, three domains (DI, DII and DIII) of EF-1α were also constructed as GST fusion proteins (Fig. 2A). All recombinant proteins were checked for their size and purity by SDS-PAGE (Fig. 2B). GST-EF-1α, GST-DI, GST-DII and GST-DIII were found to be fairly pure, and each major band showed the expected mobility. In the case of GST-α/β-tubulins, the purity was not always satisfactory. However, no appreciable degradation bands were seen in the immunoblots (Fig. 2C). Migration of GST-α-tubulin was slightly faster than expected from its molecular weight and than that of GST-β-tubulin. This is not surprising, because it is known that α-tubulin of *Tetrahymena* shows

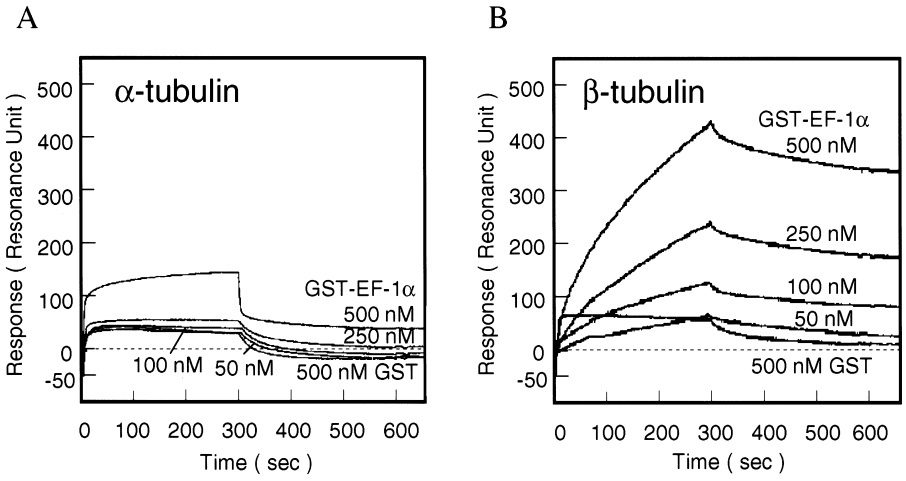

Fig. 3. EF-1α directly associates with β-tubulin but not with α-tubulin. Dose-dependent binding of GST-EF-1α to GST-α-tubulin (A) and GST-β-tubulin (B), which were immobilized on a sensor chip SA5, was analyzed by BIACORE 1000 as described in Section 2.7. Response is recorded in resonance units, proportional to the amount of the bound proteins. In each experiment, GST (500 nM) was used instead of GST-EF-1α as a negative control. Association of GST-EF-1α or GST was analyzed for 300 s, and then dissociation was observed for 300 s by washing with HBS buffer.

faster migration than β-tubulin in SDS gels, although the calculated molecular weights of the two tubulins are virtually the same [14].

Direct interactions between the purified recombinant proteins were measured with the BIACORE system, which makes possible a real-time biomolecular interaction analysis (Fig. 3). In this experiment, we immobilized biotinylated GST-α-tubulin or GST-β-tubulin on the surface of a streptavidin-coated sensor chip, and the binding of GST-EF-1α at different concentrations onto each surface was monitored. As shown in Fig. 3, a dose-dependent binding of GST-EF-1α to GST-β-tubulin, but not to GST-α-tubulin, was observed. GST alone at 500 nM did not bind to either surface. In addition, GST-EF-1α did not bind to the surface of GST alone (data not shown). Therefore, it is evident that association of GST-EF-1α with GST-β-tubulin is taking place between the EF-1α and β-tubulin moieties. Kinetic analysis revealed that the apparent dissociation constant would be in the order of 10^{-8}. However, the kinetics also suggested that the interaction is not monophasic and at least two interaction sites are involved in the interaction between EF-1α and β-tubulin.

3.3. Domains I and III of EF-1α interact with β-tubulin

It has been shown that EF-1α binds to actin in *Dictyostelium* cells through its domains I and III [19]. Thus, we next tried to identify the β-tubulin-interacting domain(s) in EF-1α. Fig. 4 shows the results of biomolecular interaction analysis done with GST-DI, GST-DII and GST-DIII. It is clearly demonstrated that GST-DI and GST-DIII bind to GST-β-tubulin, but GST-DII does not. The binding capacity of GST-DI was nearly comparable to that of GST-EF-1α, while the response of GST-DIII was significantly lower. This is due to the difference in the molecular size of these proteins. Actually, GST-DI and GST-DIII gave comparable values in their kinetic constants (see below). Importantly, however, the kinetics of the binding of these EF-1α domains seemed to be still biphasic. It suggests that there are two distinct interaction sites within β-tubulin. If we assume two sites (site 1 and site 2) of interaction in β-tubulin, dissociation constants for GST-DI and GST-DIII are estimated to be $K_D1 = 5.36 \pm 3.69 \times 10^{-9}$, $K_D2 = 1.53 \pm 0.15 \times 10^{-8}$, and $K_D1 = 2.16 \pm 1.15 \times 10^{-9}$, $K_D2 = 1.90 \pm 0.14 \times 10^{-8}$, respectively.

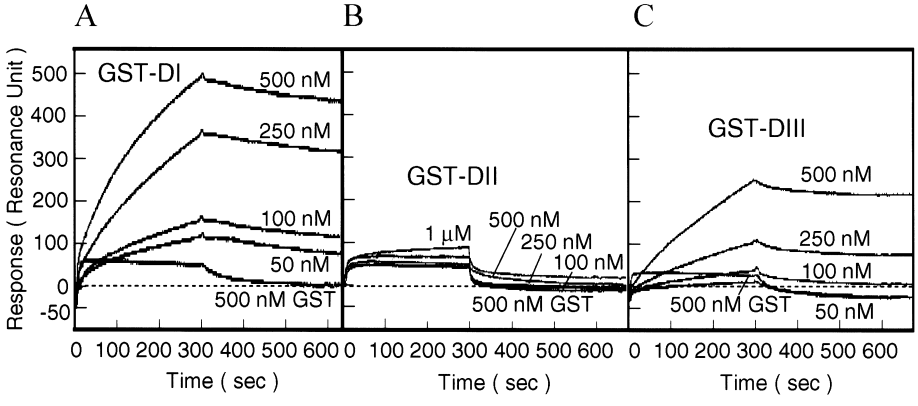

Fig. 4. GST-DI and GST-DIII bind to GST-β-tubulin, but GST-DII does not. GST fusion protein containing either DI (A), DII (B), or DIII (C) of EF-1α was analyzed for the ability to bind to GST-β-tubulin as in Fig. 3.

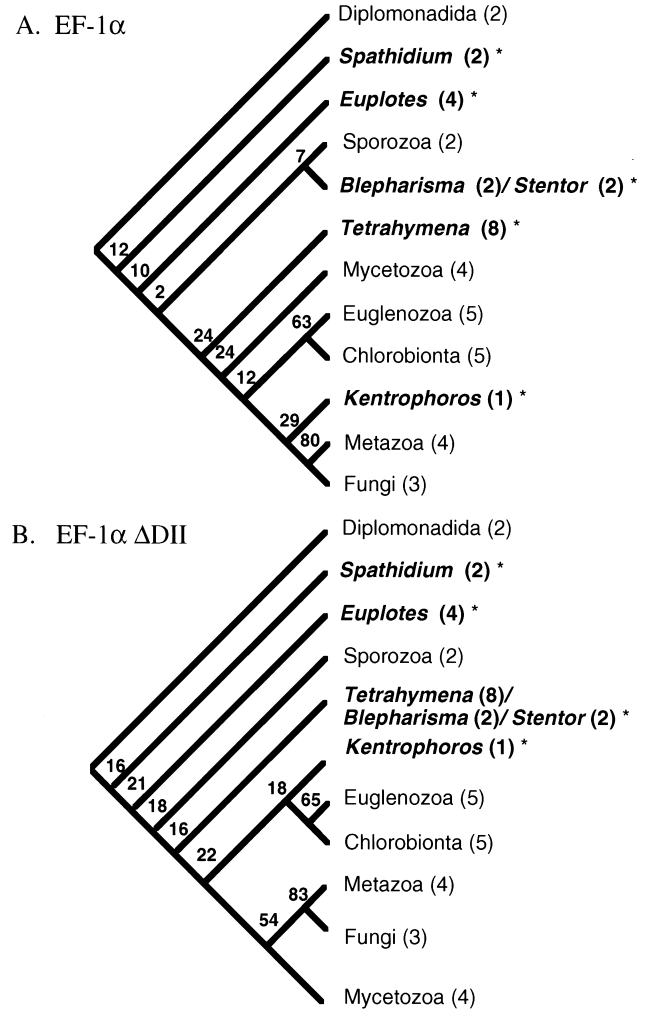

Fig. 5. Schematic representation of phylogenetic trees of EF-1α. The phylogenetic tree was inferred from either the whole sequence of EF-1α (A) or EF-1α ΔDII (B), the EF-1α sequence devoid of domain II (corresponding to residues 233–318 of *T. pyriformis* EF-1α), of 44 eukaryotes containing 19 ciliates by the method described in Section 2.8. In parentheses, the number of species used in each phylum is indicated. Numbers of each root show bootstrap values. Ciliates are represented by bold italics and marked by asterisks.

3.4. Re-evaluation of phylogeny of ciliates

The difference in tubulin binding property among the three domains of EF-1α encouraged us to construct a phylogenetic tree based on the EF-1α sequences responsible for β-tubulin binding. In Fig. 5, the phylogenetic tree inferred from the whole sequence of EF-1α (Fig. 5A) is compared with that inferred from the sequence of EF-1α composed of β-tubulin binding domains (Fig. 5B). It is interesting that the latter phylogenetic tree seems to show a tendency to a monophyletic property of Ciliophora in most of the ciliates. For example, Heterotrichea (*Blepharisma* and *Stentor*) joined to one big group together with the major group of other ciliates such as *Tetrahymena* in Fig. 5B, although an estimation value by the bootstrap method of this united group was low (16%). In contrary, Hypotrichea (*Euplotes*) and Litostomatea (*Spathidium*) insisted on making distinctive groups.

4. Discussion

In this study we have shown that EF-1α binds to tubulin in *T. pyriformis* (Fig. 1), as observed in some other organisms [5]. This property was also confirmed in vitro using recombinant proteins. Importantly, however, EF-1α binds only to β-tubulin and not to α-tubulin (Fig. 3). The reason why only β-tubulin can interact with EF-1α is not clear. Microtubules consist of non-covalently assembled one-to-one heterodimers of α/β-tubulins. These two molecules share 40% identity in their amino acid sequences, and their three-dimensional structures are basically identical [20]. Both α/β-tubulins can bind to one GTP per molecule, and GTP hydrolysis is required for the assembling of tubulins and polymerization into microtubules [21,22]. The only clear difference between the nature of the two tubulins is that hydrolysis of GTP takes place only on β-tubulin [22]. Since the microtubule-severing ability of EF-1α has been reported [7], it is conceivable that EF-1α regulates microtubule polymerization under the control of GTP hydrolysis on β-tubulin.

Domains I and III of EF-1α were found to interact with β-tubulin, while interaction of domain II was not observed (Fig. 4). It has also been reported that domains I and III of *D. discoideum* EF-1α bind to actin, but domain II does not [19]. The result is consistent with our present findings, and this interesting coincidence may indicate that EF-1α has a common feature to anchor cytoskeletal proteins. Our kinetic analysis suggested that there are two distinct binding sites on β-tubulin for each of the domains I and III of EF-1α. Identification of the EF-1α binding sites in β-tubulin will contribute to understanding the mechanism of interaction of EF-1α not only with β-tubulin but also with actin.

EF-1α appears to possess multiple functions such as in translation of mRNA and tubulin binding. Thus, in the molecule of EF-1α, there may be at least two different *covarions*, concomitantly variable codons [23]; one is a covarion derived from the function concerning translation, and another concerns cytoskeletal function. In the latter, domain II may be free from evolutionary constraint. The deviated evolutionary rates of EF-1α in ciliates and the discordance with the phylogenetic tree of rRNA might result from deviation of this latter covarion site in ciliates with respect to evolutionary constraints of tubulin. The recovery of the monophyletic state by elimination of domain II from EF-1α (Fig. 5B) suggests that domain II has evolved at different rates in ciliates. It is naturally acceptable that a constraint imposed on one molecule influences the partner molecule not to mutate. This might also be the case for the association between tubulin and EF-1α in ciliates, especially for domains I and III of EF-1α. Our phylogenetic study seems to imply that each functional domain of EF-1α has experienced a different evolutionary process.

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